

# Gene Expression in Wilms' Tumor Mimics the Earliest Committed Stage in the Metanephric Mesenchymal-Epithelial Transition

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**Wilms' tumor (WT) has been considered a prototype for arrested cellular differentiation in cancer, but previous studies have relied on selected markers. We have now performed an unbiased survey of gene expression in WTs using oligonucleotide microarrays. Statistical criteria identified 357 genes as differentially expressed between WTs and fetal kidneys. This set contained 124 matches to genes on a microarray used by Stuart and colleagues (Stuart RO, Bush KT, Nigam SK: Changes in global gene expression patterns during development and maturation of the rat kidney. Proc Natl Acad Sci USA 2001, 98:5649–5654) to establish genes with stage-specific expression in the developing rat kidney. Mapping between the two data sets showed that WTs systematically overexpressed genes corresponding to the earliest stage of metanephric development, and underexpressed genes corresponding to later stages. Automated clustering identified a smaller group of 27 genes that were highly expressed in WTs compared to fetal kidney and heterologous tumor and normal tissues. This signature set was enriched in genes encoding transcription factors. Four of these, *PAX2*, *EYA1*, *HBF2*, and *HOXA11*, are essential for cell survival and proliferation in early metanephric development, whereas others, including *SIX1*, *MOX1*, and *SALL2*, are predicted to act at this stage. *SIX1* and *SALL2* proteins were expressed in the condensing mesenchyme in normal human fetal kidneys, but were absent (*SIX1*) or reduced (*SALL2*) in cells at other developmental stages. These data imply that the blastema in WTs has progressed to the committed stage in the mesenchymal-epithelial transition, where it is partially arrested in differentiation. The WT-signature set also contained the Wnt receptor *FZD7*, the tumor antigen *PRAME*, the imprinted gene *NNAT* and the metastasis-associated transcription factor *E1AF*. (Am J Pathol 2002, 160:2181–2190)**

Wilms' tumor (WT) is a common pediatric malignancy that recapitulates the histology of the nephrogenic zone of the growing fetal kidney, and it has long been viewed as a prototype of differentiation failure in human neoplasia.<sup>2,3</sup> Certain developmentally regulated genes have already been examined in these tumors. Notable among these are *PAX2*, which is essential for kidney development,<sup>4–6</sup> and which is expressed in WTs,<sup>7,8</sup> and the *WT1* tumor suppressor, which is also essential for kidney development,<sup>9</sup> and which may function downstream of *PAX2*.<sup>10–14</sup>

To gain a more complete view of gene expression in WTs, we have performed an unbiased survey using oligonucleotide microarrays. We sought to identify first a large set of genes that are differentially expressed between WTs and control fetal kidneys and, second, a smaller and more specific subset of genes that are highly expressed in WTs relative to additional control tissues, including a histologically unrelated type of cancer. The results from the first screen statistically confirm the notion, previously based on histology and analysis of selected genes, that cells in WTs are arrested at an early stage of fetal kidney development. The identities of the smaller subset of WT signature genes emphasize high expression in WTs of genes that are essential for kidney formation and that act at the earliest committed stage in the mesenchymal-epithelial transition.

## Materials and Methods

### Tissues

WTs were an unselected series of primary nonsyndromic tumors resected at Babies Hospital of Columbia University. None of the WTs were anaplastic. Cryopreserved samples of these tumors, control fetal tissues (16- to 22-week gestations), primary Burkitt lymphomas (BLs), and pulmonary adenocarcinomas were obtained from the Columbia University Cancer Center Tissue Bank. The BL cell lines were from the American Type Culture Collection (Rockville, MD).

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### Microarrays and Probes

HG-U95A GeneChips (Affymetrix, Santa Clara, CA), which query ~10,000 genes (12,000 probe sets), were used for all analyses. The cRNA probes were synthesized as recommended by Affymetrix. Briefly, total RNA was isolated in two steps using Trizol (Invitrogen, Carlsbad, CA), followed by RNeasy (Qiagen, Valencia, CA) purification. Double-stranded cDNA was generated from 5  $\mu$ g of total RNA using a polydT oligonucleotide that contained a T7 RNA polymerase initiation site and the Superscript Choice System Kit (Invitrogen). cDNA was phenol/chloroform extracted. Biotinylated cRNA was generated by *in vitro* transcription using the Bio Array High Yield RNA Transcript Labeling System (Enzo, Farmingdale, NY). The cRNA was purified using RNeasy. cRNA was fragmented according to the Affymetrix protocol, and 15  $\mu$ g of biotinylated cRNA were hybridized to U95A microarrays (Affymetrix). After scanning, the expression values for each gene were determined using Affymetrix GeneChip software version 4.0.

### Data Analysis

Two software packages, GeneSpring (Silicon Genetics, Redwood City, CA) and Genes at Work, which utilizes the Structural Pattern Localization Analysis by Sequential Histograms, SPLASH, algorithm,<sup>15,16</sup> were used for analyzing the GeneChip data. Simple statistical and expression value cutoffs were done using the filter functions of GeneSpring. The GeneTree function, a hierarchical clustering tool in GeneSpring that creates dendrograms of genes based on the extent of relatedness of their expression patterns, was used for nonsupervised clustering. Supervised clustering of the initial set of differentially expressed genes was done using the pattern discovery algorithm of Genes at Work,<sup>15</sup> as previously described.<sup>17</sup> For the initial screen using GeneSpring (derivation of sets A and B), only those genes that gave consistent Affymetrix presence calls in the GeneChip data for both the WT and the fetal kidneys (at least five of six samples with presence calls) were analyzed. The Genes at Work algorithm, used for deriving the smaller set of WT signature genes, accepted all data, including Affymetrix absence calls. Among the WT signature genes, tissue samples for which particular probe sets gave absence calls were invariably control tissues with very low absolute signals for these probe sets. However, no significant differences were seen between the overall percentage of absence calls (all genes on the microarray) among the different tissues.

### Northern Blotting

Total RNA from WT and fetal kidneys, prepared using Trizol reagent (Invitrogen), was resolved on formaldehyde-containing agarose gels and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH). Probes for Northern blotting were partial cDNAs prepared by reverse transcriptase-polymerase chain reaction using gene-specific primers. Hybridization was in ULTRAhyb buffer (Ambion, Austin, TX) at 42°C degrees. Probes were

stripped by boiling the membranes in 0.1% sodium dodecyl sulfate/0.1 $\times$  standard saline citrate solution for 10 minutes, or were allowed to decay without stripping.

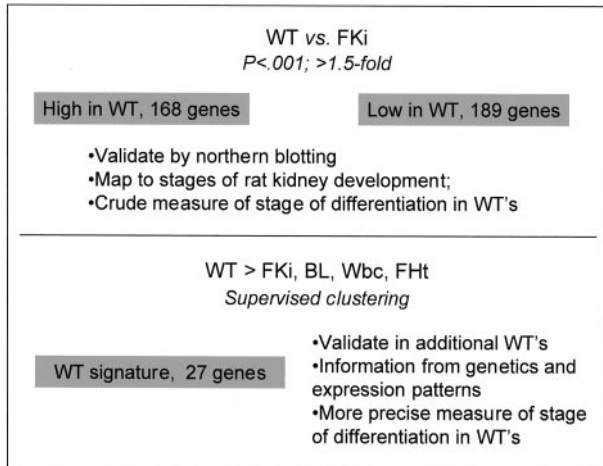
### Immunohistochemistry

Sections of WT and fetal kidneys were deparaffinized in two changes of xylene for 5 minutes each. Sections were hydrated through graded ethanols. Antigen retrieval was performed in 1 mmol/L of ethylenediaminetetraacetic acid buffer by boiling the slides in a microwave oven for 8 minutes at the maximum power and, sequentially, boiling for 15 minutes at a reduced power. Endogenous biotin was blocked by two steps of incubation for 10 to 20 minutes with egg white and 5% skim milk in 1 $\times$  Tris-buffered saline (TBS) containing 0.5% bovine serum albumin and 0.1% NaN<sub>3</sub>, respectively. Between the two blocking steps for biotin, slides were treated with 0.3% hydrogen peroxide in 0.1% NaN<sub>3</sub> to block endogenous peroxidase activity. Slides were washed three times in 1 $\times$  TBS buffer containing 0.1% Tween-20 (TBS-T) and then incubated with 5% of the blocking serum for 30 minutes in a humidified chamber. After three TBS-T washes, slides were hybridized with polyclonal antipeptide antibodies against SIX1 (Santa Cruz Biotechnology, Santa Cruz, CA), PAX2/8 (Santa Cruz), or SALL2 (Cemines, Evergreen, CO) at room temperature overnight. After washing in TBS-T, slides were incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) for 30 to 40 minutes at room temperature. Antigen-antibody complexes were developed by the Vectastain ABC kits (Vector Laboratories) and a chromogenic substrate, diaminobenzidine (DAKO, Carpinteria, CA). Sections were counterstained with hematoxylin.

### Results

#### Genes Differentially Expressed between WT and Fetal Kidneys

Nephrogenesis is an ongoing process, in which reciprocal inductive interactions of branches of the ureteric bud with overlying metanephric mesenchyme are played out repeatedly as the uretic system ramifies and the kidney grows.<sup>3,14</sup> Mid-gestation fetal kidneys are therefore a composite of fully differentiated, differentiating, and undifferentiated cells, and gene expression in whole fetal kidney is an average over these cells. WT are more homogeneous, but these neoplasms can also contain a spectrum of cell types, which are classified as blastemal, epithelial, and stromal. Despite this heterogeneity, we reasoned that an initial WT *versus* whole fetal kidney comparison could be useful in eliminating a large number of genes whose average expression did not differ between these two sources, and identifying sets of differentially expressed genes. Genes found to be overexpressed in WT by this comparison might reflect either pathological overexpression on a per-cell basis, or stage-appropriate expression in a cell type that is numerically overrepresented in WT. Both types of net overexpression are potentially interesting, and the latter category

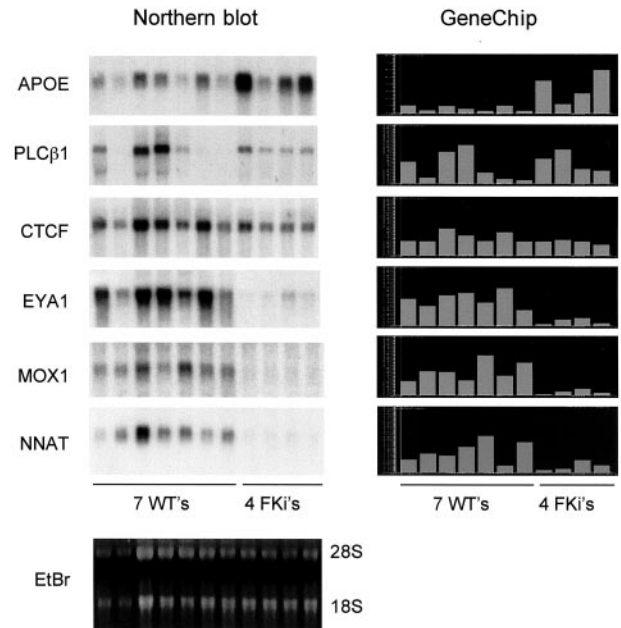


**Figure 1.** Strategy for analyzing gene expression in WTs. For a gene to be included in set A or set B, we first required a value of  $P < 0.001$  by a chi-square test for differential expression between the WTs and the control fetal kidneys. We additionally required a normalized expression value of  $> 1.5$  relative to the experiment mean in at least three samples, either WTs or fetal kidneys.

might be informative concerning the stage at which the primitive-appearing blastemal cells in WTs are blocked in their ability to differentiate.

We therefore first performed microarray analysis with cRNA probes from six sporadic (nonsyndromic) WTs, using six whole mid-gestation (16 to 22 weeks) fetal kidneys as the comparison group. We used simple statistical and expression value criteria to select an initial set of differentially expressed genes (Figure 1). This produced a set of 357 differentially expressed genes, represented by 396 oligonucleotide probes. Clustering using the GeneTree function of GeneSpring yielded two clades, with 168 genes more highly expressed in WTs than in fetal kidneys (set A), and 189 genes more highly expressed in fetal kidneys than in WTs (set B). To verify the accuracy of the microarray data, we performed Northern blotting of total RNAs from WTs and fetal kidneys and hybridized the blot successively with cDNA probes for six genes that showed various expression patterns. The Northern blotting results for each gene correlated well with the microarray data (Figure 2).

We next mapped our initial set of differentially expressed genes to all genes on the RG-U34A Affymetrix microarray that was previously used by Stuart and colleagues<sup>1</sup> in a study of normal kidney development in the rat. These authors queried gene expression in whole rat kidneys at seven successive stages of development, from 13 days postcoitum (dpc) to adulthood. Although the developing kidney is a heterogeneous mixture of cell types at each of these stages, the less differentiated cellular populations predominate at the early stages. Stuart and colleagues<sup>1</sup> used statistical criteria to establish five clusters of genes (group 1 to group 5) with characteristic temporal patterns of expression. In their classification, group 1 genes are highly expressed at the earliest stage examined, 13 dpc, a stage at which the ureteric bud has first contacted the metanephric mesenchyme, and decline in expression thereafter. Genes in the other

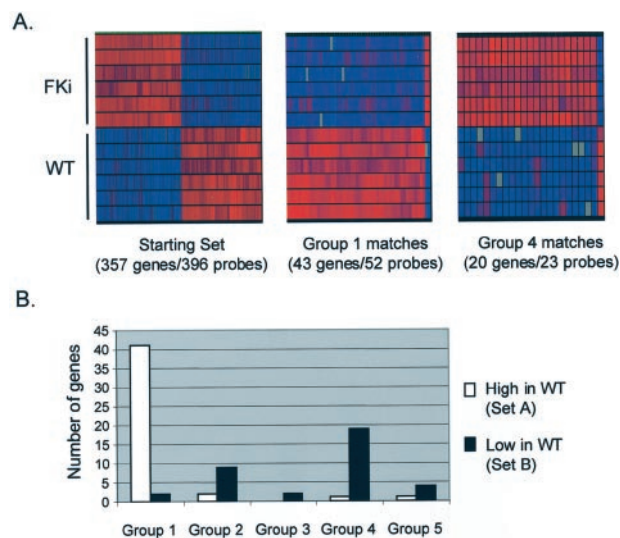


**Figure 2.** Validation of the GeneChip data by comparisons with Northern blotting. **Left:** A single Northern blot was successively hybridized with cDNA probes for the indicated genes. **Right:** The GeneChip expression data (normalized to the experiment mean) are displayed with the same order of samples. EtBr, ethidium bromide stain of the Northern gel showing 28S and 18S ribosomal RNAs.

groups peak in their expression at progressively later stages: for example, group 2 genes reach their highest expression between 17 dpc and 19 dpc, whereas group 4 genes become highly expressed only at birth (21 dpc) and group 5 genes show high expression only in fully mature adult kidneys.<sup>1</sup> We used the FASTA search algorithm as implemented in the Genetics Computer Group (GCG) package,<sup>18,19</sup> to query a database of all genes represented on the U34A GeneChip with each of the 357 differentially expressed genes in our initial set. Of these 357 genes, 124 had convincing matches by stringent criteria ( $E$  score,  $\leq 40$ ;  $Z$ -score,  $> 800$ , with evidence of orthology from inspection of the gene annotations) among the rat genes represented on the U34A GeneChip. Of these, 81 genes fell into groups 1 to 5, whereas the remainder were not detected by Stuart and colleagues<sup>1</sup> as varying significantly during development and so were not assigned to groups. Genes in our set A (high in WTs) fell overwhelmingly into group 1 (42 of 45 genes; 93%; Figure 3A), whereas genes in set B (low in WTs) fell predominantly into groups 2 to 5 (34 of 36 genes; 94%; Figure 3A). That the expression of these genes was generally consistent among the different WT cases, and among the fetal kidney controls, is shown in Figure 3B.

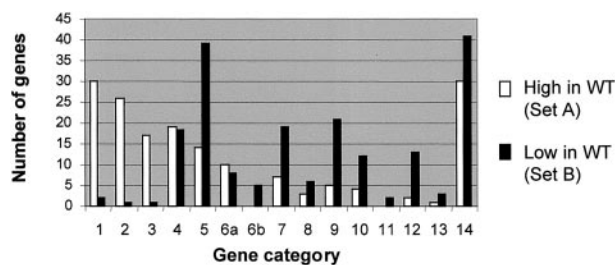
We next classified the differentially expressed genes according to known or predicted function, by reviewing sequence information and the associated scientific literature using public databases (<http://www.ncbi.nlm.nih.gov/>). We annotated each of the genes in set A and set B, and assigned them to categories similar to those used by Stuart and colleagues.<sup>1</sup> This showed that genes involved in DNA synthesis or metabolism (including repair), regulation of the cell cycle, and RNA metabolism were greatly





**Figure 3.** Correlation of gene expression in WT with normal kidney development in the rat. **A:** Genes identified in the current study that mapped to genes identified by Stuart and colleagues.<sup>1</sup> Genes that are more highly expressed in WT than in whole fetal kidneys map predominantly to the earliest group of Stuart and colleagues,<sup>1</sup> whereas genes that are expressed at lower levels in WT than in fetal kidneys map to the later groups. **B:** Graphical representations (GeneTree function, GeneSpring) showing the uniformity of relative overexpression or underexpression across multiple genes and multiple samples. Genes are arrayed along the x-axis; WT and fetal kidney samples are shown on the y-axis. Gene expression is automatically color-coded with red indicating higher and blue lower expression. Gray indicates Affymetrix absence calls. The group 1 matches are nearly all more highly expressed in WT, whereas the group 4 matches are nearly all more highly expressed in fetal kidneys. Color range is set at 1 = normal, 6 = maximum.

overrepresented in set A, whereas genes involved in several other functions were overrepresented in set B (Figure 4; gene identities in supplementary data, <http://icg.cpmc.columbia.edu/tycko/data/>). The results in terms of DNA synthesis or metabolism and regulation of the cell cycle confirm the intuitive expectation that proliferating tumor cells should overexpress genes in these categories, whereas the data for RNA metabolism are less intuitively obvious. But for all three categories, our data closely parallel those of Stuart and colleagues,<sup>1</sup> who found that genes involved in DNA synthesis or metabolism, regulation of the cell cycle, and RNA metabolism,



**Figure 4.** Functional categories of genes differentially expressed between WT and fetal kidneys. The categories are: **1**, DNA synthesis or metabolism (including repair); **2**, cell cycle regulation (including mitotic spindle); **3**, RNA metabolism (not including transcription factors); **4**, transcriptional regulation (including transcription factors); **5**, cell signaling; **6a**, protein metabolism nonproteolytic; **6b**, protein metabolism proteolytic; **7**, small molecule metabolism (enzymes); **8**, small molecule transport; **9**, extracellular matrix, adhesion, and serum proteins; **10**, intracellular trafficking; **11**, energy metabolism; **12**, cytoskeleton (not including mitotic spindle); **13**, protection against oxidative stress; **14**, unknown function.

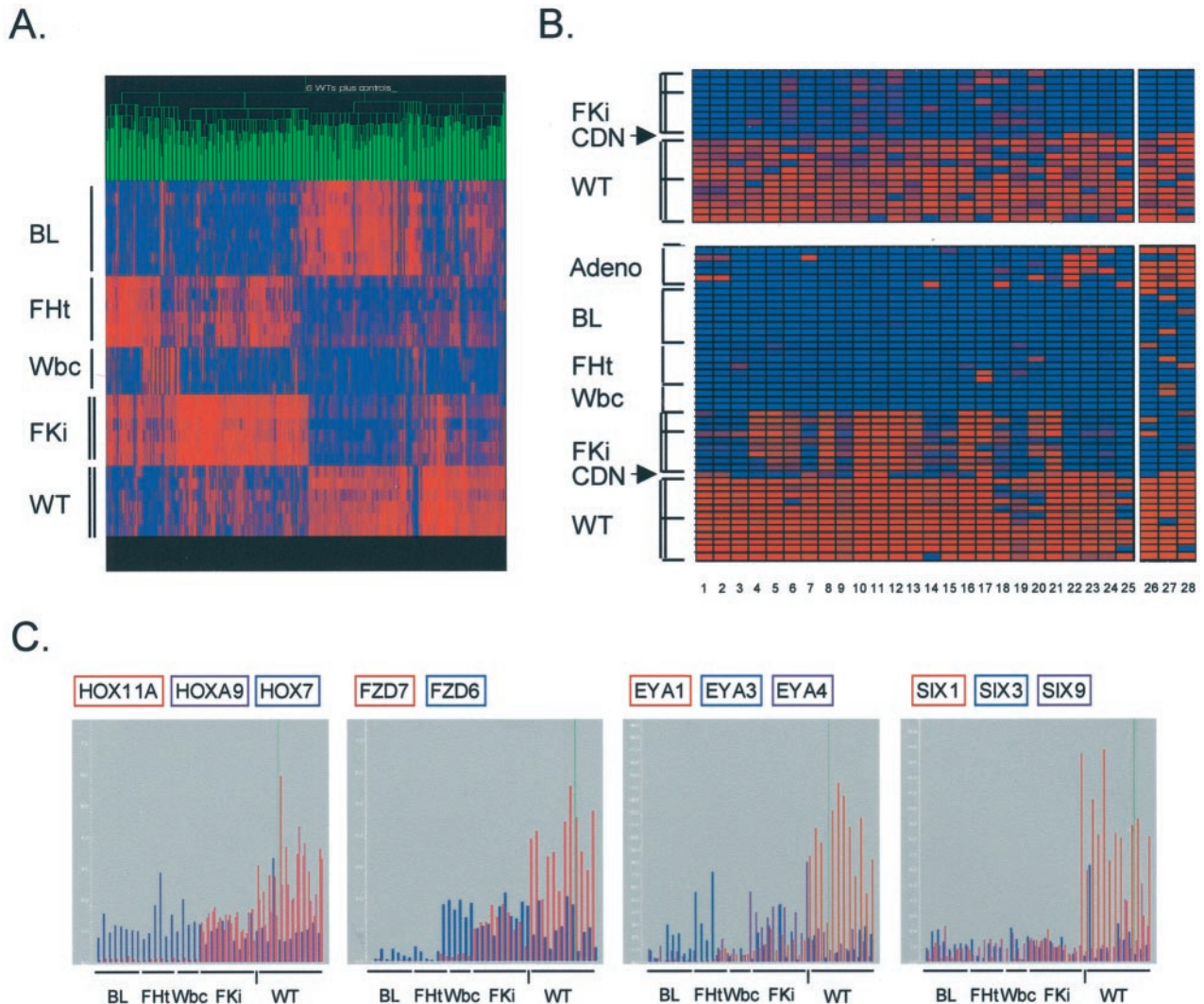
are overexpressed in the earliest stage of metanephric kidney development. Overall, these data from an unbiased screen substantiate the notion, which has previously been based on histology and selected markers, that the cells in WT are, on average, arrested in their differentiation at a point corresponding to an early stage of development of the metanephric kidney.

### Derivation of WT Signature Genes

To derive a smaller set of genes that might give more precise information about the phenotype of WT, we sought to screen-out those genes that were highly expressed as a nonspecific consequence of cellular proliferation. To do this, we added microarray data from a heterologous (histologically unrelated) highly proliferative tumor type, BL. We obtained data from a series of four primary BLs and four BL cell lines. To eliminate any genes with nonspecific housekeeping functions that may have persisted after the initial WT/fetal kidney screening, we also added heterologous normal control tissues: peripheral blood leukocytes from four healthy individuals, and six mid-gestation fetal hearts. Automated nonsupervised clustering (GeneTree function of GeneSpring) of the initial set of differentially expressed genes (set A and set B) according to their expression in these different tissues yielded patterns that clearly distinguished each type of tumor and normal tissue (Figure 5A).

More importantly, it was evident from the GeneTree display that a substantial proportion of genes in set A (high in WT) were in fact highly expressed in the primary BLs and in the BL cell lines (Figure 5A). In fact, examination of the composition of clusters containing the genes that were simultaneously high in WT and in BL showed that they contained all of the genes that fell into the DNA synthesis/repair and cell cycle categories, as well as other genes that are known to be markers of cellular proliferation. A few examples are Ki67 antigen, CDK2, cyclin B, Ku80 DNA-binding protein, and several kinesin genes. This confirmed the expectation that most of these genes would be nonspecific markers of cellular proliferation. A smaller percentage of the genes in set A were highly expressed in fetal heart and/or in peripheral blood leukocytes, but as expected these control tissues tended instead to express genes from set B (Figure 5A).

Given these observations, we were confident that we had chosen a useful combination of control tissues. We therefore used a supervised learning algorithm in the Genes at Work package,<sup>16</sup> to rank all genes based on the difference in their expression between WT and these control tissues. The 30 probe sets with the highest Z-scores (measure of probability) included 29 genes, with 1 gene (*CRABP2*) identified by two different probe sets. When this set was examined in an expanded series of WT and fetal kidneys (six additional WT and three additional fetal kidneys), all but 2 of the 29 genes showed significantly higher expression in the additional WT compared to the control tissues. The resulting 27 WT signature genes, which we refer to as set C, are shown in Figure 5B and listed in Table 1. In addition to the positive validation of these genes in additional WT, as a further



**Figure 5.** Derivation of WT signature genes. **A:** Nonsupervised clustering (GeneTree function of GeneSpring) of the 357 genes that were initially identified as differentially expressed between WTs and fetal kidneys. Many of the high in WT/low in fetal kidney genes are also high in the BLs. The color range is normal = 1, maximum = 6. All data are included, with absence calls represented by pure blue. **B:** WT signature genes. The 27 highest-scoring genes identified by SPLASH are shown here, after clustering using the GeneTree function of GeneSpring (columns 1 and 2 are two probe sets both identifying *CRABP2*). The identities of these genes are in Table 1. Tissues are: AdenoCA, pulmonary adenocarcinoma (six primary tumors); BL, Burkitt lymphoma (four primary tumors and four cell lines); FHt, mid-gestation fetal hearts (six samples); Wbc, normal peripheral blood (four samples); FKt, mid-gestation fetal kidneys (six samples in **A** and the same six samples below the dividing line in **B**, with three additional fetal kidneys above the line); CDN, differentiating cystic nephroblastoma (one case); WT, (six WTs in **A** and the same six WTs below the dividing line in **B**, with six additional WTs above the line). Tissues used for analysis by Genes at Work are **bracketed**; the adenocarcinomas were not included in deriving the WT signature genes. The genes in set C were identified by SPLASH using only data from the samples shown in **A**. The additional WTs and fetal kidneys, above the dividing lines, therefore validate the signature set. The differentiating cystic nephroblastoma shows a distinct pattern of gene expression. Because the data are normalized to all tissues, displaying the WT and fetal kidney samples alone (**top**) emphasizes the differences between them, while displaying these samples together with the heterologous tissues highlights their close affinity. Color ranges are set at 1 = normal, 6 = maximum (**top**) and 2 = normal, 10 = maximum (**bottom**). **C:** Differential expression of genes in the HOX-, FZD-, EYA-, and SIX-families. Microarray data are normalized to the experiment mean. Each bar represents the value for a single sample. BL, Burkitt lymphoma (eight samples); FHt, fetal heart (six samples); Wbc, peripheral blood leukocytes (four samples); FKt, fetal kidney (nine samples); WT, Wilms' tumor (12 samples). The **vertical dash** indicates the single case of differentiating cystic nephroblastoma.

test of specificity we examined their expression in microarray data from one case of a related but distinct type of pediatric kidney tumor, cystic differentiating nephroblastoma. This case showed a distinct pattern of gene expression, with nine of the set C genes silent, and several others detected only at low levels (Figure 5B).

### Identities of WT Signature Genes

Set C was depleted for genes that are involved directly in the general functions of DNA replication and repair and

cell-cycle control, but it was enriched for genes encoding transcription factors. The fraction of human-coding sequences devoted to transcription factors has been estimated at 6% based on genomic sequencing.<sup>20</sup> Of the genes in set A, 12% encoded known transcription factors, but 41% of genes in set C (11 of 27) were in this category (Figure 5B and Table 1). As discussed in detail below, many of the 11 transcription factor genes in set C are informative as developmental markers. Four of these genes, *PAX2*, *EYA1*, *HBF2*, and *HOXA11* have been previously shown to be essential for metanephric develop-

**Table 1.** Genes in Set C

Figure 5b	Probe set	Gene product	Symbol	GenBank
1	1057_at	Retinoic acid-binding protein II	<i>CRABP2</i>	M97815
2	41783_at	Retinoic acid-binding protein II	<i>CRABP2</i>	M97815
3	36010_at	Mox1 homeodomain protein	<i>MOX1</i>	U10492
4	39051_at	Neuronatin alpha and neuronatin beta	<i>NNAT</i>	U31767
5	37605_at	Pro-alpha1 type II collagen	<i>COL2A1</i>	L10347
6	1058_at	Brush-1; Was-family protein	<i>WASF3</i>	S69790
7	40292_at	DBCCR1; IB3089A; retinoic acid inducible	<i>DBCCR1</i>	AF027734
8	33222_at	Frizzled-7	<i>FZD7</i>	AB017365
9	37567_at	Zinc finger protein, Hsal2; KIAA0360	<i>SALL2</i>	X98834
10	37809_at	Homeobox gene; HOXA9	<i>HOXA9</i>	U41813
11	33248_at	EST matching HOXA11	<i>HOXA11</i>	H94842
12	38184_at	Pax2 paired box protein	<i>PAX2</i>	M89470
13	38952_s_at	Alpha-2 type IV collagen	<i>COL4A2</i>	M33653
14	35396_at	Hyaluronan synthase	<i>HAS2</i>	U54804
15	37073_at	EYA1C: eyes absent homolog 1	<i>EYA1</i>	AJ000098
16	38749_at	G-protein coupled receptor GPR39	<i>GPR39</i>	AI936826
17	40358_at	DNA-binding protein GLI3	<i>GLI3</i>	M57609
18	35200_at	HMG1-C high-mobility group factor	<i>HMGIC</i>	X92518
19	37283_at	MGCR gene, protein of unknown function	<i>MGCR1, MN1</i>	X82209
20	37630_at	Chordin-like gene; Unigene Hs.82223	(not assigned)	AL049176
21	38853_at	HE6; seven transmembrane-domain receptor	<i>HE6</i>	X81892
22	38545_at	Inhibin beta, B-subunit	<i>INHBB</i>	M31682
23	40004_at	SIX1 protein; homeobox protein	<i>SIX1</i>	X91868
24	41395_at	Keratan sulfate Gal-6-sulfotransferase	<i>CHST1</i>	AB003791
25	31946_s_at	HBFG-2 forkhead-family transcription factor	<i>HBFG2</i>	X74143
26	2084_s_at	E1A-F; Ets-family transcription factor	<i>E1AF</i>	D12765
27	157_at	PRAME-1 tumor antigen	<i>PRAME</i>	U65011
28	41764_at	EST similar to APOCI	<i>APOCI</i>	AA976838

ment in mice and/or humans.<sup>5,6,21-25</sup> A fifth gene in set C, *SALL2*, has not been previously implicated in kidney development, but it is homologous to *SALL1*, encoding a Spalt-family transcription factor that is essential for normal kidney development in mice and humans.<sup>26,27</sup> Some of the other transcription factor genes in set C are also likely to function in kidney development and, as discussed below, two of them, *MOX1* and *SIX1* are known to act in the *PAX/EYA* pathway.

Additional genes in set C, not encoding transcription factors, are also likely to participate in the formation of the metanephric kidney. Signaling by Wnt-family proteins, including Wnt-4, is essential for the epithelial conversion of the metanephric mesenchyme.<sup>28</sup> This occurs via interaction of Wnt ligands with one or more Frizzled-family receptors, and the *FZD7* (Frizzled-7) gene, in set C, is a candidate for this function. Interestingly, Wnt signaling is essential for appropriate expression of a chicken Spalt-family protein, cSal1.<sup>29</sup> Other genes in set C have unknown functions. One of these, neuronatin (*NNAT*), is subject to genomic imprinting, such that it is expressed only from the paternal allele.<sup>30</sup> This gene, which encodes a proteolipid, produces abundant mRNA in various fetal tissues, but is silenced on differentiation of neurons and other cell types.<sup>30,31</sup>

The *FZD7* gene, like *HOXA11*, *SIX1*, and *EYA1*, belongs to a family with multiple members, so we asked whether the microarray data could give some indication of specificity within these gene families. Using selected genes on the Hu95A array that gave consistent presence calls in one or more tissue type, it was possible to compare the expression patterns of *HOXA11* and *HOXA9* versus *HOX7*, *SIX1* versus *SIX3* and *SIX9*, *EYA1* versus *EYA3* and *EYA4*,

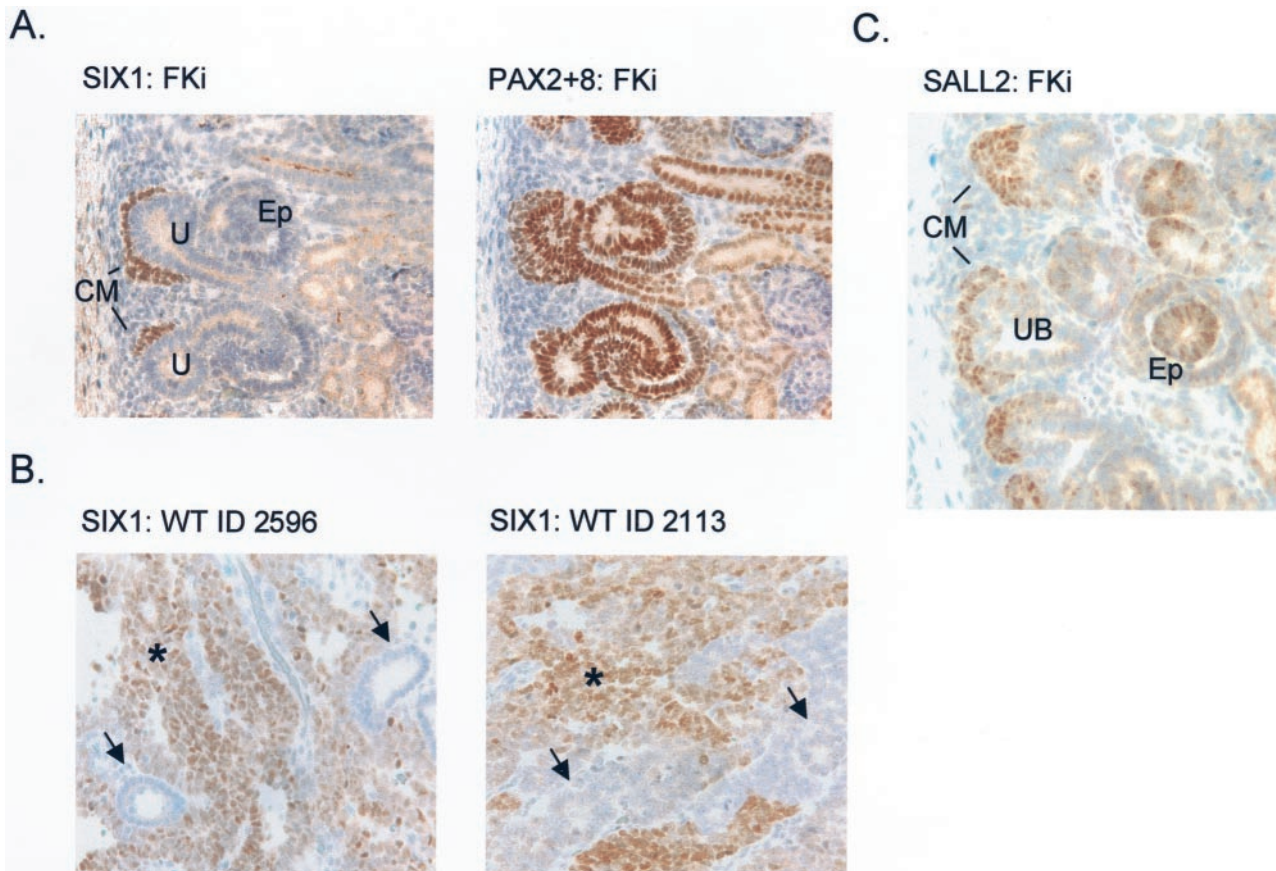
and *FZD6* versus *FZD7* (Figure 5C). These comparisons confirmed the specificity of the overexpression of *HOXA11*, *HOXA9*, *SIX1*, *EYA1*, and *FZD7* in the WTs.

Many of the set C genes are transcribed at detectable levels in fetal kidneys, whereas few are detected in the heterologous controls (Figure 5B). This confirms the intuition that there should be a close relationship between the fetal kidney and WT. However, three genes in set C, *E1AF*, *PRAME*, and *APOCI*, differed from the others in that they were expressed at high levels in WTs, but were undetectable in the normal fetal kidneys. These genes are less likely to have roles in kidney development, and more likely to be overexpressed because of neoplastic transformation per se. *E1AF* encodes an Ets-family transcription factor that is overexpressed in several types of metastatic tumor cell lines,<sup>32,33</sup> and *PRAME* encodes a cell surface antigen that is highly expressed in melanomas and acute leukemias.<sup>34,35</sup> *APOCI* produces a serum apolipoprotein. Although not expressed in BL, these genes are frequently expressed in other tumor types, as confirmed by high signals seen in our microarray data from a series of pulmonary adenocarcinomas (Figure 5B), and by queries of the Unigene database (<http://www.ncbi.nlm.nih.gov/unigene>). Several of the adenocarcinomas also expressed *SIX1* (see Discussion).

#### *Distribution of SIX1 and SALL2 Proteins in Fetal Kidneys and WTs*

The relative overexpression of particular genes in WTs compared to whole fetal kidneys might reflect either a uniform difference in expression on a per-cell basis, or a





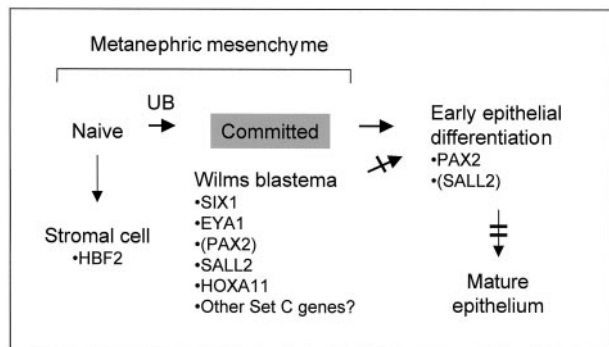
**Figure 6.** Immunohistochemical detection of SIX1 and SALL2 proteins in human fetal kidney and in WTs. **A:** Serial sections of a 20-week gestation human fetal kidney were developed with anti-SIX1 and anti-PAX2 + 8 polyclonal antisera. SIX1 is expressed only in the earliest condensing mesenchyme (metanephric blastema), which is seen directly apposed to the uretic bud-derived epithelium. PAX2 + 8 detects expression of these two proteins in the condensing mesenchyme, and there is a strong signal in differentiating epithelial structures (S-shaped and comma-shaped bodies) deeper in the kidney,<sup>8,50</sup> as well as in ureteric bud.<sup>36</sup> Weak cytoplasmic staining in differentiated epithelial cells is nonspecific (seen in control with secondary antibody alone). **B:** SIX1 is expressed in the blastemal component of WTs (asterisks), but not in the epithelial areas (arrows). This pattern was seen in three of three cases of WT examined. In these same cases the epithelial component stained strongly with anti-PAX2 + 8 (not shown). **C:** Anti-SALL2 polyclonal antiserum gives a strong and uniform signal in the condensing mesenchyme, but cells with nuclear SALL2 are also seen at lower frequencies and with somewhat reduced staining in differentiating epithelial structures. Ureteric bud derivatives are negative for SALL2. Weak cytoplasmic staining in differentiated epithelial cells is nonspecific. CM, condensing mesenchyme; U, ureteric bud branches; Ep, differentiating epithelial structures; Gl, primitive glomeruli; Tu, differentiated renal tubules.

greater numerical abundance of a particular type of cell in the WTs that might be present as minor cell population in fetal kidneys. Because we suspected that most of the genes in set C would fall into the latter category, we assessed the tissue distribution of SIX1 and SALL2 proteins in sections of WTs and normal fetal kidneys. For SIX1, the expression in fetal kidney was highly specific for the condensing mesenchyme, which was seen closely apposed to the tips of the ureteric bud branches (Figure 6A). These cells have received and responded to an inductive signal from the ureteric bud epithelium, but they do not yet show morphological transformation into epithelial structures. As expected based on previous studies,<sup>8,36</sup> a control antibody recognizing both PAX2 and PAX8 also stained these cells, but reacted more strongly with ureteric bud epithelium and with differentiating epithelial structures deeper in the developing renal cortex (Figure 6A). In two WTs examined by immunohistochemistry, SIX1 protein was detected only in the blastemal elements (Figure 7B). Like SIX1, SALL2 protein strongly marked the condensing mesenchyme, but it was also detectable at lower intensity and in fewer cells in the

differentiating epithelial structures (Figure 6C). These findings, together with published developmental information for several of the other genes in set C, indicate that for many of these genes their overexpression in WTs reflects the numerical predominance of neoplastic cells arrested at a specific developmental stage. The composition of set C can therefore be used to establish a working model for failure of cellular differentiation in WTs (Figure 7).

### Discussion

WT has long been considered a prototype for arrested cellular differentiation in cancer, but previous assessments of the WT phenotype have relied on histology and selected markers. The current data, from an unbiased survey of ~10,000 genes, support the general statement that cells in WTs are arrested in their differentiation at an early stage of metanephric development. Access to microarray data from a previous study of normal kidney development in an animal model facilitated this interpre-



**Figure 7.** Differentiation arrest in WTs inferred from gene expression profiling. A schematic of the normal metanephric differentiation program is shown. The neoplastic blastema, which comprises the bulk of many WTs, is arrested at the position indicated by the **gray box**, and relevant WT signature genes are indicated. The **single bar** indicates a partial differentiation arrest in WTs, and the **double bar** indicates a full block of differentiation. **Parentheses** indicate low but detectable expression. UB indicates the inductive interaction with the ureteric bud.

tation.<sup>1</sup> This emphasizes the benefits of using standardized methods for microarray-based studies, and making the primary data available through the Internet. A second lesson of experimental design that may be pertinent for other studies is that the direct comparison of tumor biopsies with whole-organ control samples, although it relies on averaging across heterogeneous cell populations, can still produce informative data.

Our set C was derived by an unbiased approach that identified those genes with higher expression in WTs than in the homologous tissue fetal kidney and the histologically unrelated tissues fetal heart, peripheral blood, and BL. Empirically, this set of genes was strikingly enriched in developmentally essential genes and stage-specific markers of the metanephros, and depleted in genes with more general functions. We have therefore used the operational term "WT signature" to describe this set. But few genes are expected to be completely tissue-specific, and the expression of the set C genes is not restricted to WTs. The *SIX1* gene is highly expressed in some human breast cancers and in rhabdomyosarcomas,<sup>37,38</sup> and we found high expression of *SIX1* mRNA, as well as several other genes from set C, in several pulmonary adenocarcinomas. Nor are these genes specific for normal metanephros: *Six1* mRNA is expressed in the developing somites,<sup>39</sup> and *Eya1* is essential both for kidney and for inner ear and craniofacial bone development.<sup>21</sup> By Northern blotting, human *FZD7* mRNA is most abundant in normal fetal kidney and in adult skeletal muscle, but it is also detectable in fetal lung, adult heart, and placenta.<sup>40</sup> However, in these multiple sites, these developmental regulators play homologous functions. *Eya1*, for example, is necessary for the survival of condensing mesenchyme, both in the metanephros and in the otic vesicle.<sup>21</sup>

Disproportionately represented in set C were genes in the PAX pathway, which included 4 of the 27 genes in this set. Genes in the PAX-, SIX-, MOX-, and EYA-families act together in a shared pathway for tissue growth and differentiation. EYA-family proteins bind directly to members of the SIX family of homeodomain proteins, and they provide a transcriptional co-activator function.<sup>41</sup> The ex-

pression of *Six1* and *Eya2* is induced by *Pax3* expression in murine myogenic stem cells,<sup>42</sup> paralleling genetic interactions that were originally described in *Drosophila* eye development, with *Pax* (*eyeless*) acting upstream of *Eya* (*eyes absent*), which in turn acts upstream of *Six* (*sine oculis*).<sup>43,44</sup> *Pax3* acts synergistically with *Eya2* to induce muscle differentiation,<sup>39</sup> and *Eya2* also shows synergy with *Six1* in this system.<sup>39</sup> *Eya1* is required for expression of *Six2* (and probably *Six1*) in condensing metanephric mesenchyme of the murine kidney, and for expression of *Six1* in the otic vesicle, suggesting positive feedback of *Eya/Six* heterodimers on *Six*-family genes.<sup>21</sup> There is also evidence for positive feedback of *Eya/Six* on *Pax* genes.<sup>45</sup> The *Mox1* and *Mox2* proteins bind directly to *Pax1* and *Pax3* proteins, respectively.<sup>46</sup> *Mox2* is essential for myogenesis in mice,<sup>47</sup> but it is not yet known whether *Mox1* is essential for kidney development.

Mice mutant for *Pax2* fail to develop normal kidneys<sup>6</sup> and provide an exaggerated model for the human haploinsufficiency disorder renal-coloboma syndrome. Similarly, deletion or mutation of *Eya1* in the mouse germline caused defective morphogenesis in the kidney.<sup>21,23</sup> These mice are severe models for another human disorder, the brachio-oto-renal (BOR) syndrome, which is because of *EYA1* haploinsufficiency and which is associated with renal dysgenesis, inner ear defects, and craniofacial abnormalities.<sup>22</sup>

Although there is no evidence that it acts in the PAX pathway, the *Sall1* gene, closely related to *Sall2*, is also essential for kidney development.<sup>27</sup> Mouse embryos lacking this gene are an exaggerated model for a human genetic syndrome, Townes-Brocks syndrome, that is associated with renal hypoplasia and other developmental abnormalities because of heterozygosity for mutations in *SALL1*.<sup>26</sup> *SALL2* was included in set C, but our analysis did not identify *SALL1* in either set A or set C, even though this gene has been reported as overexpressed in WTs.<sup>48</sup> A probe set querying *SALL1* is present on the HG-U95A microarray, and examination of the data showed that this gene is indeed highly expressed in some, but not all, WTs (not shown). This intertumor variability explains the lack of inclusion of this gene in set C.

Optimally, conclusions about the stage of differentiation in WTs should be based on large panels of markers. Our analysis has identified a well-studied marker, *PAX2*, and several new ones with informative expression patterns in the normal fetal kidney. *Eya1* is expressed only at the earliest stage of commitment of the metanephric mesenchyme to the epithelial-mesenchymal transition, as indicated by *in situ* hybridization in developing mouse kidneys.<sup>21,49</sup> *Pax2* is also most highly expressed in this early condensing mesenchyme, but in both mouse and human *Pax2* mRNA is also detected at lower levels in later mesenchyme-derived structures (comma- and S-shaped-bodies) and in ureteric bud.<sup>8,50</sup> Like *Eya1* and, as we have shown, *SIX1*, the *Hoxa11* gene is a specific marker for the earliest committed metanephric mesenchyme.<sup>25</sup> The murine *Bf2* gene (human orthologue *HBF2*) is expressed in the primitive stromal cells of the metanephric kidney,<sup>24</sup> so this gene does not fit the pattern of *Pax2*, *Eya1*, *Hoxa11*, and *Six1*. In this regard, it may be relevant



that of the genes in set C, *HBF2* showed the greatest variability in expression among the different cases of WT (Figure 5B). This may be because WTs can contain varying proportions of stromal-lineage cells, but two technical problems, namely variable composition in different areas of the same tumor and lack of an effective anti-HBF2 antibody, have prevented us from testing this.

Although we have focused on transcription factors, a number of the other genes in set C are likely to affect the mesenchymal-epithelial transition. The Wnt pathway and frizzled-family receptors, such as *FZD7*, were mentioned above. Another crucial signaling pathway in metanephric development is mediated by ligands in the bone morphogenetic protein (Bmp) family and requires sulfated glycosaminoglycans in the extracellular matrix.<sup>51,52</sup> So, the presence of a carbohydrate sulfotransferase gene, *CHST1*, in set C is not surprising.

A working model for differentiation arrest in WTs, shown in Figure 7, is consistent with the available data. In this model, WTs arise from a primitive mesenchymal cell that is capable of differentiating in the stromal and epithelial directions. A differentiation block, which is partial, is present at the transition from committed mesenchyme to early epithelium. This is consistent with the accumulation in most WTs of an abundant blastemal component that expresses *PAX2*, *SIX1*, *EYA1*, *SALL2*, and *HOXA11*. Primitive epithelial structures can form in WTs from the cells that succeed in passing this block, but these are often a minority of cells. Cadherin genes are useful markers for the successive stages of epithelial differentiation, with cadherin-11 being widely expressed in both naïve and committed metanephric mesenchyme, and cadherin-6 and E-cadherin expressed in successively more mature epithelial structures.<sup>53</sup> E-cadherin appeared in our set B (supplementary data), and consistent with our model, the microarray data ranks the expression of these genes in WTs in the order cadherin-11 > cadherin-6 > E-cadherin (data not shown).

What might be the functional importance of the signature genes that we have identified? There is no evidence that genes in set C play a primary role as proto-oncogenes in initiating Wilms' tumorigenesis, but they may have essential downstream functions. In particular, because mutations of several of these genes cause renal hypoplasia or aplasia because of failed proliferation of nonneoplastic metanephric mesenchyme, and because additional set C genes act coordinately in these same pathways, these genes may also be required to maintain cell survival and proliferation in the blastema of WTs.

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### References

1. Stuart RO, Bush KT, Nigam SK: Changes in global gene expression patterns during development and maturation of the rat kidney. *Proc Natl Acad Sci USA* 2001, 98:5649–5654
2. Miyagawa K, Kent J, Schedl A, van Heyningen V, Hastie ND: Wilms' tumour—a case of disrupted development. *J Cell Sci* 1994, 18:S1–S5
3. Davies JA, Perera AD, Walker CL: Mechanisms of epithelial development and neoplasia in the metanephric kidney. *Int J Dev Biol* 1999, 43:473–478
4. Torres M, Gomez-Pardo E, Dressler GR, Gruss P: Pax-2 controls multiple steps of urogenital development. *Development* 1995, 121:4057–4065
5. Sanyanusin P, McNoe LA, Sullivan MJ, Weaver RG, Eccles MR: Mutation of PAX2 in two siblings with renal-coloboma syndrome. *Hum Mol Genet* 1995, 4:2183–2184
6. Favor J, Sandulache R, Neuhauser-Klaus A, Pretsch W, Chatterjee B, Senft E, Wurst W, Blanquet V, Grimes P, Sporle R, Schughart K: The mouse Pax2(1Neu) mutation is identical to a human PAX2 mutation in a family with renal-coloboma syndrome and results in developmental defects of the brain, ear, eye, and kidney. *Proc Natl Acad Sci USA* 1996, 93:13870–13875
7. Dressler GR, Douglass EC: Pax-2 is a DNA-binding protein expressed in embryonic kidney and Wilms tumor. *Proc Natl Acad Sci USA* 1992, 89:1179–1183
8. Eccles MR, Yun K, Reeve AE, Fidler AE: Comparative in situ hybridization analysis of PAX2, PAX8, and WT1 gene transcription in human fetal kidney and Wilms' tumors. *Am J Pathol* 1995, 146:40–45
9. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R: WT-1 is required for early kidney development. *Cell* 1993, 74:679–691
10. Torban E, Goodyer PR: Effects of PAX2 expression in a human fetal kidney (HEK293) cell line. *Biochim Biophys Acta* 1998, 1401:53–62
11. Dehbi M, Ghahremani M, Lechner M, Dressler G, Pelletier J: The paired-box transcription factor, PAX2, positively modulates expression of the Wilms' tumor suppressor gene (WT1). *Oncogene* 1996, 13:447–453
12. McConnell MJ, Cunliffe HE, Chua LJ, Ward TA, Eccles MR: Differential regulation of the human Wilms tumour suppressor gene (WT1) promoter by two isoforms of PAX2. *Oncogene* 1997, 14:2689–2700
13. Donovan MJ, Natoli TA, Sainio K, Amstutz A, Jaenisch R, Sariola H, Kreidberg JA: Initial differentiation of the metanephric mesenchyme is independent of WT1 and the ureteric bud. *Dev Genet* 1999, 24:252–262
14. Schedl A, Hastie ND: Cross-talk in kidney development. *Curr Opin Genet Dev* 2000, 10:543–549
15. Califano A: SPLASH: structural pattern localization analysis by sequential histograms. *Bioinformatics* 2000, 16:341–357
16. Califano A, Stolovitzky G, Tu Y: Analysis of gene expression microarrays for phenotype classification. *Proc Int Conf Intell Syst Mol Biol* 2000, 8:75–85
17. Klein U, Tu Y, Stolovitzky G, Mattioli M, Cattoretti G, Husson H, Freedman A, Inghirami G, Cro L, Baldini L, Neri A, Califano A, Dalla-Favera R: Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med* 2001, 194:1625–1638
18. Pearson WR: Rapid and sensitive sequence comparison with FASTP and FASTA. San Diego, Academic Press 1990, pp 63–98
19. Genetics Computer Group: Wisconsin Package, version 10.0. Madison, 1999
20. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Hudson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Miklos GLG, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di Francesco V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV, Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang ZY, Wang A, Wang X, Wang J, Wei MH, Wides R, Xiao C, Yan C, Yao A, Ye J, Zhan M, Zhang W, Zhang H, Zhao Q, Zheng L, Zhong F,

- Zhong W, Zhu SC, Zhao S, Gilbert D, Baumhueter S, Spier G, Carter C, Cravchik A, Woodage T, Ali F, An H, Awe A, Baldwin D, Baden H, Barnstead M, Barrow I, Beeson K, Busam D, Carver A, Center A, Cheng ML, Curry L, Danaher S, Davenport L, Desilets R, Dietz S, Dodson K, Doup L, Ferreira S, Garg N, Gluecksmann A, Hart B, Haynes J, Haynes C, Heiner C, Hladun S, Hostin D, Houck J, Howland T, Ibegwam C, Johnson J, Kalush F, Kline L, Koduru S, Love A, Mann F, May D, McCawley S, McIntosh T, McMullen I, Moy M, Moy L, Murphy B, Nelson K, Pfannkoch C, Pratts E, Puri V, Qureshi H, Reardon M, Rodriguez R, Rogers YH, Romblad D, Ruhfel B, Scott R, Sitter C, Smallwood M, Stewart E, Strong R, Suh E, Thomas R, Tint NN, Tse S, Vech C, Wang G, Wetter J, Williams S, Williams M, Windsor S, Winn-Deen E, Wolfe K, Zaveri K, Abril JF, Guigó R, Campbell MJ, Sjolander KV, Karlak B, Kejarawal A, Mi H, Lazareva B, Hatton T, Narechania A, Diemer K, Muruganujan A, Guo N, Sato S, Bafna V, Istrail S, Lippert R, Schwartz R, Walenz B, Yooshep S, Allen D, Basu A, Baxendale J, Blick L, Caminha M, Carnes-Stine J, Caulk P, Chiang YH, Coyne M, Dahlke B, Mays AD, Dombroski M, Donnelly M, Ely D, Esparham S, Fosler C, Gire H, Glanowski S, Glasser K, Glodek A, Gorokhov M, Graham K, Gropman B, Harris M, Heil J, Henderson S, Hoover J, Jennings D, Jordan C, Jordan J, Kasha J, Kagan L, Kraft C, Levitsky A, Lewis M, Liu X, Lopez J, Ma D, Majoros W, McDaniel J, Murphy S, Newman M, Nguyen T, Nguyen N, Nodell M, Pan S, Peck J, Peterson M, Rowe W, Sanders R, Scott J, Simpson M, Smith T, Sprague A, Stockwell T, Turner R, Venter E, Wang M, Wen M, Wu D, Wu M, Xia A, Zandieh A, Zhu X: The sequence of the human genome. *Science* 2001, 291:1304–1351
21. Xu PX, Adams J, Peters H, Brown MC, Heaney S, Maas R: Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat Genet* 1999, 23:113–117
22. Abdelhak S, Kalatzis V, Heilig R, Compain S, Samson D, Vincent C, Weil D, Cruaud C, Sahly I, Leibovici M, Bitner-Glindzic M, Francis M, Lacombe D, Vigneron J, Charachon R, Boven K, Bedbeder P, Van Regenmortel N, Weissenbach J, Petit C: A human homologue of the *Drosophila* eyes absent gene underlies branchio-oto-renal (BOR) syndrome and identifies a novel gene family. *Nat Genet* 1997, 15:157–164
23. Johnson KR, Cook SA, Erway LC, Matthews AN, Sanford LP, Paradies NE, Friedman RA: Inner ear and kidney anomalies caused by IAP insertion in an intron of the *Eya1* gene in a mouse model of BOR syndrome. *Hum Mol Genet* 1999, 8:645–653
24. Hatini V, Huh SO, Herzlinger D, Soares VC, Lai E: Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged helix transcription factor *BF-2*. *Genes Dev* 1996, 10:1467–1478
25. Patterson LT, Pembaur M, Potter SS: *Hoxa11* and *Hoxd11* regulate branching morphogenesis of the ureteric bud in the developing kidney. *Development* 2001, 128:2153–2161
26. Kohlhase J, Wischermann A, Reichenbach H, Froster U, Engel W: Mutations in the *SALL1* putative transcription factor gene cause Townes-Brocks syndrome. *Nat Genet* 1998, 18:81–83
27. Nishinakamura R, Matsumoto Y, Nakao K, Nakamura K, Sato A, Copeland NG, Gilbert DJ, Jenkins NA, Scully S, Lacey DL, Katsuki M, Asashima M, Yokota T: Murine homolog of *SALL1* is essential for ureteric bud invasion in kidney development. *Development* 2001, 128:3105–3115
28. Kispert A, Vainio S, McMahon AP: *Wnt-4* is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* 1998, 125:4225–4234
29. Farrell ER, Munsterberg AE: *csal1* is controlled by a combination of FGF and Wnt signals in developing limb buds. *Dev Biol* 2000, 225:447–458
30. John RM, Aparicio SA, Ainscough JF, Arney KL, Khosla S, Hawker K, Hilton KJ, Barton SC, Surani MA: Imprinted expression of neuronatin from modified BAC transgenes reveals regulation by distinct and distant enhancers. *Dev Biol* 2001, 236:387–399
31. Joseph R, Tsang W, Dou D, Nelson K, Edvardsen K: Neuronatin mRNA in PC12 cells: downregulation by nerve growth factor. *Brain Res* 1996, 738:32–38
32. Hida K, Shindoh M, Yoshida K, Kudoh A, Furaoka K, Kohgo T, Fujinaga K, Totsuka Y: Expression of E1AF, an ets-family transcription factor, is correlated with the invasive phenotype of oral squamous cell carcinoma. *Oral Oncol* 1997, 33:426–430
33. Habelhah H, Okada F, Kobayashi M, Nakai K, Choi S, Hamada J, Moriuchi T, Kaya M, Yoshida K, Fujinaga K, Hosokawa M: Increased E1AF expression in mouse fibrosarcoma promotes metastasis through induction of MT1-MMP expression. *Oncogene* 1999, 18:1771–1776
34. Ikeda H, Lethe B, Lehmann F, van Baren N, Baurain JF, de Smet C, Chambost H, Vitale M, Moretta A, Boon T, Coulie PG: Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor. *Immunity* 1997, 6:199–208
35. van Baren N, Chambost H, Ferrant A, Michaux L, Ikeda H, Millard I, Olive D, Boon T, Coulie PG: PRAME, a gene encoding an antigen recognized on a human melanoma by cytolytic T cells, is expressed in acute leukaemia cells. *Br J Haematol* 1998, 102:1376–1379
36. Poleev A, Fickenscher H, Mundlos S, Winterpacht A, Zabel B, Fidler A, Gruss P, Plachov D: PAX8, a human paired box gene: isolation and expression in developing thyroid, kidney and Wilms' tumors. *Development* 1992, 116:611–623
37. Ford HL, Landesman-Bollag E, Dacwag CS, Stukenberg PT, Pardee AB, Seldin DC: Cell cycle-regulated phosphorylation of the human Six1 homeodomain protein. *J Biol Chem* 2000, 275:22245–22254
38. Khan J, Bittner ML, Saal LH, Teichmann U, Azorsa DO, Gooden GC, Pavan WJ, Trent JM, Meltzer PS: cDNA microarrays detect activation of a myogenic transcription program by the PAX3-FKHR fusion oncogene. *Proc Natl Acad Sci USA* 1999, 96:13264–13269
39. Heanue TA, Reshef R, Davis RJ, Mardon G, Oliver G, Tomarev S, Lassar AB, Tabin CJ: Synergistic regulation of vertebrate muscle development by *Dach2*, *Eya2*, and *Six1*, homologs of genes required for *Drosophila* eye formation. *Genes Dev* 1999, 13:3231–3243
40. Sagara N, Toda G, Hirai M, Terada M, Katoh M: Molecular cloning, differential expression, and chromosomal localization of human *frizzled-1*, *frizzled-2*, and *frizzled-7*. *Biochem Biophys Res Commun* 1998, 252:117–122
41. Ohto H, Kamada S, Tago K, Tominaga SI, Ozaki H, Sato S, Kawakami K: Cooperation of six and *eya* in activation of their target genes through nuclear translocation of *Eya*. *Mol Cell Biol* 1999, 19:6815–6824
42. Ridgeway AG, Skerjanc IS: Pax3 is essential for skeletal myogenesis and the expression of *Six1* and *Eya2*. *J Biol Chem* 2001, 276:19033–19039
43. Halder G, Callaerts P, Flister S, Walldorf U, Kloter U, Gehring WJ: Eyeless initiates the expression of both *sine oculis* and *eyes absent* during *Drosophila* compound eye development. *Development* 1998, 125:2181–2191
44. Niimi T, Seimiya M, Kloter U, Flister S, Gehring WJ: Direct regulatory interaction of the eyeless protein with an eye-specific enhancer in the *sine oculis* gene during eye induction in *Drosophila*. *Development* 1999, 126:2253–2260
45. Pignoni F, Hu B, Zavitz KH, Xiao J, Garrity PA, Zipursky SL: The eye-specification proteins *So* and *Eya* form a complex and regulate multiple steps in *Drosophila* eye development. *Cell* 1997, 91:881–891
46. Stamatakis D, Kastriaki M, Mankoo BS, Pachnis V, Karagozeos D: Homeodomain proteins *Mox1* and *Mox2* associate with *Pax1* and *Pax3* transcription factors. *FEBS Lett* 2001, 499:274–278
47. Mankoo BS, Collins NS, Ashby P, Grigorieva E, Pevny LH, Candia A, Wright CV, Rigby PW, Pachnis V: *Mox2* is a component of the genetic hierarchy controlling limb muscle development. *Nature* 1999, 400:69–73
48. Ma Y, Singer DB, Gozman A, Ford D, Chai L, Steinhoff MM, Hansen K, Maizel AL: *Hsal 1* is related to kidney and gonad development and is expressed in Wilms tumor. *Pediatr Nephrol* 2001, 16:701–709
49. Kalatzis V, Sahly I, El-Amraoui A, Petit C: *Eya1* expression in the developing ear and kidney: towards the understanding of the pathogenesis of branchio-oto-renal (BOR) syndrome. *Dev Dyn* 1998, 213:486–499
50. Winyard PJ, Risdon RA, Sams VR, Dressler GR, Woolf AS: The PAX2 transcription factor is expressed in cystic and hyperproliferative dysplastic epithelia in human kidney malformations. *J Clin Invest* 1996, 98:451–459
51. Bullock SL, Fletcher JM, Beddington RS, Wilson VA: Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev* 1998, 12:1894–1906
52. Miyazaki Y, Oshima K, Fogo A, Hogan BL, Ichikawa I: Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. *J Clin Invest* 2000, 105:863–873
53. Cho EA, Patterson LT, Brookhiser WT, Mah S, Kintner C, Dressler GR: Differential expression and function of cadherin-6 during renal epithelium development. *Development* 1998, 125:803–812